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October 9, 2004

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Date

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Method for the diagnosis of inflammatory diseases and  
infections with determination of LASP-1  
immunoreactivity

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5 The present invention relates to novel uses of the  
protein LASP-1 and any closely related proteins which,  
at least with regard to their first 200 amino acids,  
have substantially the same sequence as LASP-1 and  
hence a comparable immunoreactivity, and of fragments  
10 thereof, for the medical diagnosis of inflammatory  
diseases and infections, in particular of sepsis and  
sepsis-like systemic infections and of inflammatory  
diseases which are localized in the brain or which  
extend to the brain. It is based on the detection for  
15 the first time of greatly increased concentrations of  
the protein LASP-1 in the brain tissue of primates in  
which a sepsis or systemic inflammation was induced

experimentally by toxin administration, and on the subsequent detection of a greatly increased LASP-1 immunoreactivity in the circulation of patients suffering from sepsis and of Alzheimer patients, and an  
5 increased corresponding immunoreactivity also being found in patients suffering from cardiac infarction.

Where, in the following description, the term LASP-1 is used, this term is intended to denote not only the  
10 known protein LASP-1 having the specific amino acid sequence specified in SEQ ID NO:1 but also any related proteins which have an identical sequence to this LASP-1 over wide ranges, for example the protein LAP-1, explanatory statements about such proteins appearing at  
15 the end of the experimental section of this Application. This definition applies to all references to LASP-1, unless something else is evident from the specific context for a person skilled in the art. When the term LASP-1 is used, this term also means fragments  
20 having an LASP-1 immunoreactivity, it being possible for all stated products to be present both in free form and optionally in a form bound to binding proteins and/or in a posttranslationally modified form, for example in glycosylated and/or phosphorylated form.

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The present invention has its origin in intensive research work by the Applicant in relation to further improvements of the diagnosis and therapy of inflammations and infections.

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Inflammations are defined very generally as certain physiological reactions of an organism to different types of external effects, such as, for example,

injuries, burns, allergens, infections by microorganisms, such as bacteria and fungi and viruses, to foreign tissues which trigger rejection reactions, or to certain endogenous states of the body which trigger inflammation, for example in autoimmune diseases and cancer. Inflammations may occur as harmless, localized reactions of the body but are also typical features of numerous serious chronic and acute diseases of individual tissues, organs, organ parts and tissue parts.

Local inflammations are generally part of the healthy immune response of the body to harmful effects, and hence part of the life-preserving defence mechanism of the organism. However, if inflammations are part of a misdirected response of the body to certain endogenous processes, such as, for example, in autoimmune diseases, and/or are of a chronic nature, or if they reach systemic extents, as in the case of systemic inflammatory response syndrome (SIRS) or in a severe sepsis caused by infection, the physiological processes typical of inflammatory reactions go out of control and become the actual, frequently life-threatening pathological process. In the context of the present Patent Application, Alzheimer's disease, which is characterized by an unstoppable progressive corticocerebral atrophy occurring as a rule in the 5th to 6th decade of life and, owing to its frequency, is of major economic importance, is also regarded as a disease which is accompanied by typical symptoms of an inflammation, in particular of the brain.

It is now known that the origin and the course of

inflammatory processes are controlled by a considerable number of substances which are predominantly of a protein or peptide nature, or are accompanied by the occurrence of certain biomolecules for a more or less limited time. The endogenous substances involved in inflammatory reactions include in particular those which can be assigned to the cytokines, mediators, vasoactive substances, acute phase proteins and/or hormonal regulators. The inflammatory reaction is a complex physiological reaction in which both endogenous substances activating the inflammatory process (e.g.  $\text{TNF-}\alpha$ , interleukin-1) and deactivating substances (e.g. interleukin-10) are involved.

In systemic inflammations, as in the case of sepsis or of septic shock, the inflammation-specific reaction cascades spread in an uncontrolled manner over the whole body and become life-threatening in the context of an excessive immune response. Regarding the current knowledge about the occurrence and the possible role of individual groups of endogenous inflammation-specific substances, reference is made, for example, to A. Beishuizen et al., "Endogenous Mediators in Sepsis and Septic Shock", *Advances in Clinical Chemistry*, Vol. 33, 1999, 55-131; and C. Gabay et al., "Acute Phase Proteins and Other Systemic Responses to Inflammation", *The New England Journal of Medicine*, Vol. 340, No. 6, 1999, 448-454. Since the understanding of sepsis and related systemic inflammatory diseases, and hence also recognized definitions, have changed in recent years, reference is also made to K. Reinhart et al., "Sepsis und septischer Schock" [Sepsis and Septic Shock], in: *Intensiv-medicin*, Georg Thieme Verlag,

Stuttgart, New York, 2001, 756-760, where a modern definition of sepsis is given. In the context of the present Application, the terms sepsis and inflammatory diseases used are based on the definitions as given in the three stated references.

Whereas at least in Europe the systemic bacterial infection detectable by a positive blood culture long characterized the term sepsis, sepsis is now primarily understood as being systemic inflammation which is caused by infection but, as a pathological process, has considerable similarities with systemic inflammations which are triggered by other causes. Said transformation in the understanding of sepsis has resulted in changes in the diagnostic approaches. Thus, the direct detection of bacterial pathogens was replaced or supplemented by complex monitoring of physiological parameters and, more recently, in particular by the detection of certain endogenous substances involved in the sepsis process or in the inflammatory process, i.e. specific "biomarkers".

Of the large number of mediators and acute phase proteins which are known or presumed to be involved in an inflammatory process, the ones which are suitable for diagnostic purposes are in particular those whose occurrence is very specific for inflammatory diseases or certain phases or severities of inflammatory diseases, whose concentrations change in a dramatic and diagnostically significant manner and which moreover have the stabilities required for routine determinations and reach high concentration values. For diagnostic purposes, the reliable correlation of

pathological process (inflammation, sepsis) with the respective biomarker is of primary importance (good specificity and selectivity, without there being any need to know its role in the complex cascade of the endogenous substances involved in the inflammatory process.

A known endogenous substance which is particularly suitable as a sepsis biomarker is procalcitonin. Procalcitonin is a prohormone whose serum concentrations reach very high values under the conditions of a systemic inflammation of infectious aetiology (sepsis), whereas it is virtually undetectable in healthy persons. High values of procalcitonin are also reached in a relatively early stage of a sepsis so that the determination of procalcitonin is also suitable for early diagnosis of a sepsis or for early distinguishing of a sepsis caused by infection from severe inflammations which have other causes. The determination of procalcitonin as a sepsis marker is the subject of the publication by M. Assicot et al., "High serum procalcitonin concentrations in patients with sepsis and infection", The Lancet, Vol. 341, No. 8844, 1993, 515-518; and the patents DE 42 27 454 C2 and EP 0 656 121 B1 and US 5,639,617. Reference is hereby made to said patents and to early literature references mentioned in said publications for supplementing the present description. In recent years, the number of publications on the subject of procalcitonin has greatly increased. As typical of recent published reviews, reference is also therefore made to W. Karzai et al., "Procalcitonin - A New Indicator of the Systemic Response to Severe

Infection", Infection, Vol. 25, 1997, 329-334; and M. Oczenski et al., "Procalcitonin: a new parameter for the diagnosis of bacterial infection in the peri-operative period", European Journal of Anaesthesiology 5 1998, 15, 202-209; and furthermore H. Redl et al., "Procalcitonin release patterns in a baboon model of trauma and sepsis: Relationship to cytokines and neopterin", Crit Care Med 2000, Vol. 28, No. 11, 3659-3663; and H. Redl et al., "Non-Human Primate Models of 10 Sepsis", in: Sepsis 1998; 2:243-253; and the further literature references cited therein.

The availability of the sepsis marker procalcitonin has given considerable impetus to sepsis research, and 15 intensive efforts are now being made to find further biomarkers which can supplement the procalcitonin determination and/or are capable of providing additional information for purposes of fine diagnosis or differential diagnosis. The search for potential 20 novel sepsis biomarkers is, however, complicated by the fact that frequently very little or nothing is known about the exact function or about the exact reasons for the occurrence of certain endogenous substances which are involved in the inflammatory or sepsis process.

25 The results of the experimental testing of a fruitful purely hypothetical approach to the determination of further potential sepsis markers are to be found in DE 198 47 690 A1 and WO 00/22439. There, it is shown 30 that, in the case of sepsis, not only is the concentration of the prohormone procalcitonin is increased but also significantly increased concentrations can be observed for other peptide



substances which have prohormone immunoreactivity. While the phenomenon described is well documented, the causes of the increase in the prohormone immunoreactivity in sepsis are still substantially  
5 unexplained.

In the present Application, the results of another fruitful, purely experimental approach in the search for further inflammation- or sepsis-specific  
10 biomolecules is reported. These experimental investigations, too, originate from the determination of procalcitonin in relation to systemic inflammatory reactions of infectious aetiology. Thus, it had been observed at a very early stage that, in sepsis, the  
15 procalcitonin is evidently not formed in the same way as when it is a precursor for the hormone calcitonin. Thus, high procalcitonin levels were also observed in patients whose thyroid had been removed. The thyroid therefore cannot be the organ in which procalcitonin is  
20 formed or secreted during sepsis. In the publications by H. Redl et al., "Procalcitonin release patterns in a baboon model of trauma and sepsis: Relationship to cytokines and neopterin", Crit Care Med 2000, Vol. 28, No. 11, 3659-3663; and H. Redl et al., "Non-Human  
25 Primate Models of Sepsis", Sepsis 1998; 2:243-253, the results of experimental investigations which are intended to clarify the formation of procalcitonin in sepsis are reported. In said papers, an artificial sepsis is induced by endotoxin administration to  
30 primates (baboons), and the experimentally induced states in which the highest procalcitonin concentrations in the blood are reached are determined. In the context of the present Application, a further

development of the experimental animal model described in said papers serves for determining novel endogenous inflammation- and/or sepsis-specific biomarkers of a peptide or protein nature, the occurrence of which is characteristic of sepsis or certain forms of inflammation and/or sepsis and which therefore permit a specific, optionally organ-specific, sepsis or inflammation diagnosis. The primate model was chosen on the basis of the very considerable similarity of the physiology of primates and humans and the high cross-reactivity with many therapeutic and diagnostic human reagents.

Since the endogenous substances formed during inflammations are part of the complex reaction cascade of the body, not only are such substances also of diagnostic interest but attempts are also currently being made, with considerable effort, to intervene therapeutically in the inflammatory process by influencing the origin and/or the concentration of individual substances of this type, in order to stop at as early a stage as possible the systemic spread of the inflammation which is observed, for example, in sepsis. In this context, endogenous substances which can be shown to be involved in the inflammatory process are also to be regarded as potential therapeutic targets. Attempts starting from certain mediators of the inflammatory process to positively influence said process therapeutically are described, for example, in E.A. Panacek, "Anti-TNF strategies", Journal für Anästhesie und Intensivbehandlung; No. 2, 2001, 4-5; T. Calandra et al., "Protection from septic shock by neutralization of macrophage migration inhibitory

factor", Nature Medicine, Vol. 6, No. 2, 2000, 164-170;  
or K. Garber, "Protein C may be sepsis solution",  
Nature Biotechnology, Vol. 18, 2000, 917-918. In view  
of the fairly disappointing therapeutic approaches to  
5 date, there is considerable interest in identifying  
endogenous biomolecules which, are as inflammation- or  
sepsis-specific as possible and, as therapeutic  
targets, also open up new prospects for success for the  
therapeutic control of inflammation.

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The present invention is based on the fact that, in  
primates and humans, the greatly overexpressed protein  
LASP-1 or a protein which is identical to it over large  
N-terminal sequence segments is detectable in a soluble  
15 form in inflammations caused by infections in  
cytoplasmic brain tissue, in particular in contrast to  
healthy persons or untreated persons in whom it is not  
found or is found only in concentrations at the  
analytical limit of detection, but also in contrast to  
20 other body tissues of septic primate experimental  
animals, making this protein appear suitable for  
diagnosing inflammation and diagnosing infection or  
sepsis where the brain is involved.

25 The invention is furthermore based on the fact that it  
was found that substantially increased LASP-1  
immunoreactivity is measurable also in the circulation  
of human sepsis patients, but also of Alzheimer  
patients, underlining or greatly enhancing the value of  
30 the knowledge gained on the basis of brain extracts of  
primates.

The uses in diagnosis, which arise owing to the

detection for the first time of the brain-specific occurrence of LASP-1 in the experimental simulation of inflammations on sepsis and the detection of a substantially increased LASP-1 immunoreactivity in the circulation, are described in more detail below.

Claims 1 to 17 define the resulting novel methods according to the invention, and preferred embodiments thereof.

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Claim 18 relates to the use of proteins having LASP-1 immunoreactivity, or specific binding partners of such proteins, for therapeutic purposes.

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As mentioned in more detail below in the experimental section, the starting point of the invention was the finding that, after experimental triggering of an artificial sepsis in baboons by endotoxin administration (LPS from *Salmonella Typhimurium*) and

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working-up of brain tissue of the treated animals by 2D gel electrophoresis, a protein which was substantially identical, and in particular at least over the segment of the amino acids from 1 to 200, to the known protein LASP-1 was found as one of the

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products identifiable only in the treated animals, the protein moreover being observed in considerably increased concentration only in the brain tissue. For

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its identification, a new protein spot, which was found only in the brain tissue of the treated animals and, according to gel electrophoresis, had a molecular weight of about  $36 \pm 3$  kDa and an isoelectric point of about 6.6-7.0, was isolated from the electrophoresis gel and separated into fragments by trypsin digestion,

said fragments being analyzed in a manner known per se by mass spectrometry. By comparison with the known mass spectrum of the trypsin-treated protein LASP-1, the protein of the isolated spot was identified as a protein which was identical to the protein LASP-1 at least over wide segments.

The identification of the product found in the protein spot is described in more detail in the experimental section, and the identification described is to be regarded, according to recognized principles of interpretation, as the safe identification of a product having an amino acid sequence of the protein LASP-1.

The theoretically determined molecular weight of the peptide chain of LASP-1 (cf. SEQ ID NO:1; 261 amino acids) is 29,717 Dalton. LASP-1 is, however, present physiologically in a posttranslationally processed form (glycosylated and phosphorylated). Thus, the product subsequently defined as LASP-1 was originally identified as a 40 kDa cAMP-dependent phosphoprotein ("pp40") in stomach wall cells (cf. C.S. Chew et al., Journal of Cell Science 113, 2035-2045 (2000); C.S. Chew et al., Am. J. Physiol. 275 (Cell Physiol. 44): C56-C67, (1998)).

The amino acid sequence of the phosphoprotein defined as LASP-1 was originally determined as that of the putative express product of the cDNA of a gene "MLN 50" from the range q11-q21.3 of chromosome 17, which gene is overexpressed in human breast cancer cell lines (C. Tomasetto et al., FEBS Letters 373 (1995): 245-249; C. Tomasetto et al., Genomics 28: 367-376 (1995);

I. Bièche et al., Cancer Res. 56: 3886-3890 (1996)).  
The name LASP-1 is attributable to the fact that, in  
this protein, for the first time a so-called LIM domain  
(a Zn-binding domain known from various proteins; cf.  
5 A. Hammarström et al., Biochemistry 1996; 35, 12723-  
12732) is combined with a domain (SH3) known from the  
receptor-independent so-called src kinase, a tyrosine  
kinase (LIM and SH3 Protein); cf. also Brian K. Kay et  
al., FASEB J. 14, 231-241 (2000).

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LASP-1 mRNA is detectable everywhere in normal cells  
(e.g. prostate, liver, muscle, brain, various cell  
types) and is overexpressed in a certain percentage of  
breast cancer cases. It was found that LASP-1 is an  
15 actin-binding protein which is assumed to belong to the  
proteins associated with the cytoskeleton and to be  
important for the cell shape and cell mobility and  
possibly the signal transmission. It is also detectable  
in nerve endings. LASP-1 is solubilized by detergents  
20 only with difficulty. In vitro, LASP-1 has proved to be  
a substrate which can be outstandingly phosphorylated  
with the cAMP-dependent serine-threonine kinase PKA.  
LASP-1 has different expression patterns in different  
epithelium cell types (cf. C.S. Chew et al., Journal of  
25 Cell Science 113, 2035-2045 (2000); C.S. Chew et al.,  
Am. J. Physiol. 275 (Cell Physiol. 44): C56-C67,  
(1998)).

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The occurrence of LASP-1 or of a protein substantially  
corresponding thereto in overexpressed form in the  
brain tissue in sepsis is of considerable scientific,  
diagnostic and possibly also therapeutic interest. The  
fact that LASP-1 is a kinase substrate and

phosphoprotein lends it a certain similarity to the so-called tau-proteins, which play a role, particularly for diagnostic purposes, in Alzheimer's disease (cf. for example G.A. Jicha et al., Journal of  
5 Neurochemistry, 69, 2087-2095 (1997) and the literature cited therein; and EP 637 418; EP 737 208; EP 772 634; EP 610 330; and EP 618 968).

It is now well known that Alzheimer's disease can be  
10 regarded as a brain-specific inflammatory process without there being any intention thereby to make a statement regarding the cause of the disease or consequence of the disease (Lih-Fen Lue et al., GLIA 35:72-79, 2001; Michael Hüll et al., DDT Vol. 4, No. 6:  
15 275-282; (June 1999); F. Licastro et al., Journal of Neuroimmunology 103 (2000), 97-102; Neuroinflammation Working Group: Haruhiko Akiyama et al., Neurobiology of Aging 21 (2000) 383-421; Michael Hüll et al., Exp. Opin. Invest. Drugs (2000) 9(4):671-683; M. Hüll et  
20 al., Eur Arch Psychiatry Clin Neurosci (1996) 246:124-128; J. Rhodin et al., Annals New York Academy of Sciences, 199x, 345-352). The fact that the phosphoprotein LASP-1 or a protein substantially corresponding thereto is detectable in overexpressed  
25 form specifically in the brain under experimental conditions which simulate a sepsis or a systemic inflammation process made it very probable that overexpression of LASP-1 or of a certain expression form of LASP-1 is observable also in Alzheimer's  
30 disease, and the determination of LASP-1 is therefore also of importance for diagnosing Alzheimer' disease. Subsequently performed immunodiagnostic determinations of an immunoreactivity in sera of sepsis and Alzheimer

patients which was assignable to the sequence of the first 200 amino acids of LASP-1 confirmed this assumption experimentally in a convincing, impressive manner.

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The publications to date on LASP-1 by no means indicate such a possibility. On the basis of the findings available to date, it was not to be expected that the physiological concentrations of LASP-1 in the brain and  
10 also in the circulation as a consequence of sepsis and/or brain-specific inflammation, such as Alzheimer's disease, have changed significantly and detectably, and therefore a determination of LASP-1 concentrations in the course of a sepsis process or of an inflammatory  
15 brain disease might be of diagnostic interest.

The detection, according to the invention, of comparatively high LASP-1 concentrations in the brain tissue of primates in which an artificial sepsis was  
20 induced by toxin administration, together with the simultaneous impossibility of detecting LASP-1 in otherwise completely identically treated samples of control animals, or in other tissue samples of septic animals, and the subsequent immunodiagnostic detection  
25 in the human circulation, is highly significant. Since the occurrence was observable only in the treated animals, in particular only a relatively short time after sepsis was induced by toxin administration, it is possible to make use of this fact for providing a  
30 promising diagnostic sepsis, infection and inflammation diagnosis method by determination of LASP-1 immunoreactivity. Moreover, the proven suitability of LASP-1 as a diagnostic marker and prognosis marker for



Alzheimer's disease is of particular interest.

The determination of LASP-1 can be carried out by any arbitrary suitable assay method, but the determination  
5 in a body fluid, including liquor (cerebrospinal fluid) of a patient by an immunodiagnostic route (by means of an immunoassay) using suitable selective antibodies appears most advantageous from practical points of view.

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Owing to the fact that, when sepsis is induced experimentally in the brain of primates and subsequently also in the circulation of human patients, an increased occurrence of LASP-1 or of a protein  
15 substantially identical thereto could be detected for the first time, the possibility of using the LASP-1 immunoreactivity in particular for diagnostic purposes in association with inflammations and infections which manifest themselves in the brain is thus created. For  
20 this purpose, it is possible to prepare LASP-1 or suitable partial peptides thereof or epitopes or epitope combinations specifically by a synthetic or genetic engineering method, optionally also by processes which are now part of the prior art. LASP-1  
25 partial peptides, optionally in marked form, may also be required as calibrators, tracers and competitors for certain assay formats for immunodiagnostic determinations and are prepared for this purpose in the manner explained. Certain currently preferred specific  
30 embodiments are described in more detail in the following experimental section.

Furthermore, LASP-1 fragments or suitable partial

sequences thereof can be used according to known modern state-of-the-art methods also for producing specific polyclonal or monoclonal antibodies which are suitable as auxiliaries for the diagnostic determination of LASP-1 in body fluids of a patient and/or also as potential therapeutic agents. Certain currently preferred specific polyclonal affinity-purified anti-LASP-1 antibodies are described in more detail in the following experimental section. The production of suitable monoclonal antibodies against known peptide partial sequences is now part of the general prior art and need not be described particularly. Furthermore, antibody production using techniques of direct genetic immunization with a corresponding DNA should also be mentioned expressly. It is therefore within the scope of the present invention to use, for example, a cDNA of LASP-1 or LASP-1 fragments for the immunization, since it has been found in the past that the spectrum of the obtainable antibodies can be extended with the use of such immunization techniques. However, it is also possible to use known antibodies against LASP-1 (cf. for example V. Schreiber et al., *Molecular Medicine* 4: 675-687, 1998; C.S. Chew et al., *Am. J. Physiol.* 275 (Cell Physiol. 44): C56-C67, (1998)).

Since, in the course of the work described here, an LASP-1 product which has a molar mass of about 36 kDa and has a solubility sufficient for the determination described was found in the gel of the 2D electrophoresis, it could not be ruled out that the LASP-1 product found is an expression product differing from the actual LASP-1 described in the literature, for example the product of an alternative splicing, and/or

has a certain type of glycosylation and/or phosphorylation, which imparts to it the observed solubility and on the basis of which it differs significantly from the 40 kDa product isolated from the stomach wall. It was therefore considered from the start of the investigations as being within the scope of the present invention to carry out the determination of LASP-1 with the aid of a specific assay in which specific partial sequences and/or optionally also glycosylation and/or phosphorylation patterns are detected. The preparation of antibodies, in particular monoclonal antibodies, which recognize specific glycosylations and/or phosphorylations is in principle known and is described, for example, in connection with the determination of certain phosphorylation forms of the so-called tau-proteins as part of the diagnosis of Alzheimer's disease.

In the immunological determination of LASP-1, it is possible in principle to proceed as described, for example, for the selective procalcitonin determination in P.P. Ghillani et al., "Monoclonal antipeptide antibodies as tools to dissect closely related gene products", The Journal of Immunology, Vol. 141, No. 9, 1988, 3156-3163; and P.P. Ghillani et al., "Identification and Measurement of Calcitonin Precursors in Serum of Patients with Malignant Diseases", Cancer Research, Vol. 49, No. 23, 1989, 6845-6851. Variations of the techniques described and/or further immunization techniques can be found by a person skilled in the art in relevant standard works and publications and applied in context.

LASP-1 or optionally LASP-1 fragments or products having a partially identical sequence, such as LAP-1, can, on the basis of the results available, serve as immunoreactivity or specific marker peptides (biomarkers) for the diagnosis and for the monitoring of inflammations and infections (in particular of systemic infections of the sepsis type and inflammations of the brain, such as Alzheimer's disease). Like the determination of procalcitonin, the determination of LASP-1 can be performed for the early differential diagnosis and for the detection and for the preparation of a prognosis, for assessment of the severity and for therapy-accompanying assessment of the course of inflammations and infections, the content of LASP-1 being determined in such a method, preferably in a sample of a biological fluid, including the so-called liquor, or optionally also of a tissue of a patient, and conclusions being drawn about the presence of an inflammation involving the brain or of a sepsis from the determined presence and/or amount of LASP-1, and the result obtained being correlated with the severity of the sepsis and optionally the possibilities of treatment and/or the prospects of treatment being estimated.

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A determination of LASP-1 as part of a multiparameter determination in which at least one further inflammation or infection parameter is determined simultaneously and in which a measured result in the form of a set of at least two measured quantities is obtained and is evaluated for the fine diagnosis of the inflammation or infection appears to be of particular interest. Further inflammation or infection parameters

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of this type are considered to be those which are selected from the group consisting of the parameters which are partly known or are disclosed in the prior or parallel Patent Applications of the Applicant, which group consists of procalcitonin, CA 125, CA 19-9, S100B, S100A proteins, soluble cytokeratin fragments, in particular CYFRA 21, TPS and/or soluble cytokeratin-1 fragments (sCY1F), the peptides inflammin and CHP, peptide prohormones, glycine N-acyltransferase (GNAT), carbamoyl phosphate synthetase 1 (CPS 1) and the fragments thereof and the C-reactive protein (CRP) or fragments of all said proteins. It is advantageous to carry out the multiparameter determination as a simultaneous determination by means of a chip technology measuring apparatus or an immunochromatographic measuring apparatus, in which the evaluation of the complex measured result obtained using the measuring apparatus is carried out with the aid of a computer program.

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In a preferred embodiment, the method is carried out as a heterogeneous sandwich immunoassay in which one of the antibodies is immobilized on an arbitrary solid phase, for example the walls of coated test tubes (for example consisting of polystyrene; "coated tubes"; CT) or on microtitre plates, for example consisting of polystyrene, or on particles, for example magnetic particles, while the other antibody carries a residue which represents a directly detectable label or permits selective linkage with a label and serves for detection of the sandwich structures formed. Delayed or subsequent immobilization using suitable solid phases is also possible.

In principle, it is possible to use all marking techniques which can be used in assays of the type described, including marking with radio isotopes, enzymes or fluorescent, chemoluminescent or bioluminescent labels and directly optically detectable colour markings, such as, for example, gold atoms and stain particles, as used in particular for point-of-care (POC) or accelerated tests. In the case of heterogeneous sandwich immunoassays, the two antibodies may also have parts of a detection system of the type described below in association with homogeneous assays.

It is therefore within the scope of the present invention to design the method according to the invention also as an accelerated test.

The method according to the invention can furthermore be designed as a homogeneous method in which the sandwich complexes formed from the two antibodies and the LASP-1 to be detected remain suspended in the liquid phase. In such a case, it is preferable to mark both antibodies with parts of a detection system, which then permits generation of a signal or triggering of a signal when both antibodies are integrated into a single sandwich. Such techniques can be designed in particular as fluorescence amplification or fluorescence extinction detection methods. A particularly preferred method of this type relates to the use of detection reagents to be employed in pairs, as described, for example, in US-A-4 822 733, EP-B1-180 492 or EP-B1-539 477 and the prior art cited therein. They permit a measurement which selectively measures only reaction products which contain the two

marking components in a single immune complex, directly in the reaction mixture. As an example, reference may be made to the technology offered under the trade names TRACE<sup>®</sup> (Time Resolved Amplified Cryptate Emission) or KRYPTOR<sup>®</sup>, which realizes the teachings of the above-mentioned Applications.

Below, the discovery and identification of LASP-1 and the determination thereof in biological fluids (sera or plasmas) of patients suffering from various diseases are described in more detail. The figures show the following:

Fig. 1 Views of 2D electrophoresis gels which permit a comparison of the spot pattern of cytoplasmic brain proteins of a healthy baboon (A) with the brain proteins of a baboon 5 h after a sepsis induced by LPS administration (B). The arrow indicates the positions of the sepsis-specific product according to the invention (LASP-1), which is highlighted by a circle in diagram (B).

Fig. 2 The mass spectrum of the trypsin-digested product isolated from the gel of the 2D gel electrophoresis.

Fig. 3 A comparative representation of the results of the measurement of the LASP-1 (1-200) immunoreactivity using immunoassays with different pairs of three antibodies, which were used in solid phase-bound or marked form and which recognized amino acid sequences

121-137, 147-159 and 170-187 of LASP-1. It can be seen that substantially identical results were obtained with all pairs.

5 Fig. 4 The results of an immunodiagnostic determination of an LASP-1 (1-200) immunoreactivity in sera of 294 healthy control persons and of 96 patients suffering from sepsis.

10 Fig. 5 The results of an immunodiagnostic determination of an LASP-1 (1-200) immunoreactivity in sera of 294 healthy control persons and of 80 patients suffering from Alzheimer's disease.

15 Fig. 6 The results of an immunodiagnostic determination of an LASP-1 (1-200) immunoreactivity in sera of 294 healthy control persons and of 20 patients suffering from cardiac infarction.

## EXPERIMENTAL SECTION

### 25 1. Infection simulation by endotoxin administration in an animal model (baboons).

On the basis of the experiments carried out with baboons for the stimulation of procalcitonin secretion by endotoxin injections (cf. H. Redl et al.,  
30 "Procalcitonin release patterns in a baboon model of trauma and sepsis: Relationship to cytokines and neopterin", Crit Care Med 2000, Vol. 28, No. 11, 3659-



3663; H. Redl et al., "Non-Human Primate Models of Sepsis", in: Sepsis 1998; 2:243-253), baboons (male, about 2 years old, weighing from 27 to 29 kg) were each intravenously administered 100  $\mu$ g of LPS (lipopolysaccharide from Salmonella Typhimurium, source: Sigma) per kg body weight. From 5 to 5.5 h after the injection, the animals were sacrificed by intravenous administration of 10 ml of dolethal. Within 60 min of their death, all organs/tissues were dissected and were stabilized by freezing in liquid nitrogen.

During the further processing, 1.5 ml of buffer A (50 mM Tris/HCl, pH 7.1, 100 mM KCl, 20% of glycerol) were added to samples of the individual frozen tissues (1 g) while cooling with nitrogen, and the samples were pulverized in a porcelain mortar to give a powder (cf. J. Klose, "Fractionated Extraction of Total Tissue Proteins from Mouse and Human for 2-D Electrophoresis", in: Methods in Molecular Biology, Vol. 112: 2-D Proteome Analysis Protocols, Humana Press Inc., Totowa, NJ). After subsequent centrifuging for 1 hour at 100,000 g and +4°C, the supernatant obtained was recovered and was stored at -80°C until required for further processing.

## 2. Proteome analysis using cytoplasmic brain proteins of baboons.

Cytoplasmic brain cell protein extracts of, on the one  
5 hand, healthy baboons (control) and, on the other hand,  
baboons which had been injected with LPS were used in a  
proteome analysis. In the initial analytical 2D gel  
electrophoresis, brain extract containing 100  $\mu$ g of  
10 protein was stabilized to 9M urea, 70 mM DTT, 2%  
ampholyte pH 2-4 and then separated by means of  
analytical 2D gel electrophoresis, as described in  
J. Klose et al., "Two-dimensional electrophoresis of  
proteins: An updated protocol and implications for a  
functional analysis of the genome", Electrophoresis  
15 1995, 16, 1034-1059. The visualization of the proteins  
in the 2D electrophoresis gel was effected by means of  
silver staining (cf. J. Heukeshoven et al., "Improved  
silver staining procedure for fast staining in Phast-  
System Development Unit. I. Staining of sodium dodecyl  
20 gels", Electrophoresis 1988, 9, 28-32).

For evaluation, the protein spot patterns of the  
samples of treated animals were compared with the  
protein spot patterns which resulted from brain tissue  
25 samples of untreated animals. Furthermore, the protein  
spot patterns from the brain tissue of treated animals  
were also compared with those of other tissues of the  
same treated animals (the results are not shown  
individually). Substances which occurred in no control  
30 sample but additionally in all treated animals were  
selected for further analytical investigations. Fig. 1  
shows a comparison of the 2D electrophoresis gels for a  
control sample (A) and a sample of a treated animal

(B), the additional protein spot in (B) corresponding to a novel protein whose position is highlighted by an arrow and a circle.

5 The novel specific proteins identified in the protein spot pattern of the analytical 2D gel electrophoresis were then prepared by means of preparative 2D gel electrophoresis using 350  $\mu$ g of protein (once again cf. (10)). In the preparative 2D gel electrophoresis, the  
10 staining was effected by means of Coomassie Brilliant Blue G250 (cf. V. Neuhoff et al., "Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and  
15 R-250", Electrophoresis 1988, 9, 255-262).

The protein spots preselected for the further analysis were cut out of the gel, using the method which is described in A. Otto et al., "Identification of human  
20 myocardial proteins separated by two-dimensional electrophoresis using an effective sample preparation for mass spectrometry", Electrophoresis 1996, 17, 1643-1650, trypsin-digested and then analyzed by means of mass spectrometry, in particular with the use of mass  
25 spectrometric investigations as described and discussed, for example in G. Neubauer et al., "Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex", in: nature genetics, vol. 20, 1998, 46-50;  
30 J. Lingner et al., "Reverse Transcriptase Motifs in the Catalytic Subunit of Telomerase", in: Science, Vol. 276, 1997, 561-567; M. Mann et al., "Use of mass spectrometry-derived data to annotate nucleotide and

protein sequence databases", in: TRENDS in Biochemical Sciences, Vol. 26, 1, 2001, 54-61. The trypsin-digested samples were subjected to MALDI-TOF mass spectrometry.

### 5    3.    Identification of LASP-1

As shown in Figures 1(A) and 1(B), brain tissue extracts of baboons to which an LPS injection had been administered contain, inter alia, a spot of a protein  
10 for which a molecular weight of approx. 36 kDa  $\pm$  3 kDa was estimated on the basis of the gel electrophoresis data in comparison with marker substances of known molecular weight, while an isoelectric point of approx. 6.6-7.0 was determined from the relative position of  
15 the protein from the first dimension.

In the mass analysis of the trypsin digestion of the novel protein spot, an attempt at assignment to peptides was undertaken for 21 fragments on the basis  
20 of database information. The result was that twelve of the assignments obtained corresponded to fragments of the protein chain of the phosphoprotein LASP-1 (SEQ ID NO:1). The so-called "sequence coverage" was 38%, which, according to the recognized criteria  
25 customary in the field, is to be regarded as unambiguous identification of the protein contained in the spot as the protein chain of LASP-1.

Thus, for example, it was possible to assign the  
30 following mass fragments to the LASP-1 protein chain (SEQ ID NO:1): 1202.714; 1254.615; 1291.745; 1360.813; 1418.898; 1443.959; 1489.826; 1551.943; 1604.040; 1608.960; in particular, the assignment according to

MS-Fit 3.3.1. Protein Prospector 3.4.1., Database  
NCBIno. 7.25.2002 was as follows:

5 The mass 1202.714 ( $MH^+$ ) corresponds to the sequence  
QQSELQSQVR (SEQ ID NO:2). The theoretical molecular  
weight of this fragment is 1201.604. In the amino acid  
sequence of LASP-1, this sequence occurs at the  
positions 76-95.

10 The mass 1254.615 corresponds to the sequence ACFHCETCK  
(SEQ ID NO:3). The theoretical molecular weight of this  
fragment is 1253.498. In the amino acid sequence of  
LASP-1, this sequence occurs at the positions 28-36.

15 The mass 1291.745 corresponds to the sequence  
KPYCNAHYPK (SEQ ID NO:4). The theoretical molecular  
weight of this fragment is 1290.617. In the amino acid  
sequence of LASP-1, this sequence occurs at the  
positions 50-59.

20 The mass 1360.813 corresponds to the sequence  
VNCLDKFWHK (SEQ ID NO:5). The theoretical molecular  
weight of this fragment is 1359.675. In the amino acid  
sequence of LASP-1, this sequence occurs at the  
25 positions 18-27.

The mass 1418.898 corresponds to the sequence  
GFSVVADTPELQR (SEQ ID NO:6). The theoretical molecular  
weight of this fragment is 1417.719. In the amino acid  
30 sequence of LASP-1, this sequence occurs at the  
positions 97-109.

The mass 1443.959 corresponds to the sequence

LKQQSELSQSVR (SEQ ID NO:7). The theoretical molecular weight of this fragment is 1442.783. In the amino acid sequence of LASP-1, this sequence occurs at the positions 74-85.

5

The mass 1489.826 corresponds to the sequence MGPSGGEGMEPERR (SEQ ID NO:8). The theoretical molecular weight of this fragment is 1488.644. In the amino acid sequence of LASP-1, this sequence occurs at the positions 131-144.

10

The mass 1551.943 was assigned to the sequence TGDTGMLPANYVEAI (SEQ ID NO:9). The theoretical molecular weight of this fragment is 1550.728. In the amino acid sequence of LASP-1, this sequence occurs at the positions 247-261.

15

The mass 1604.040 corresponds to the sequence GKGFSVVADTPELQR (SEQ ID NO:10). The theoretical molecular weight of this fragment is 1602.836. In the amino acid sequence of LASP-1, this sequence occurs at the positions 95-109.

20

The mass 1608.960 corresponds to the sequence QSFTMVADTPENLR (SEQ ID NO:11). The theoretical molecular weight of this fragment is 1607.761. In the amino acid sequence of LASP-1, this sequence occurs at the positions 60-73.

25

Nine of the ten identified fragments described occur in the segment of the amino acids 1-200 of LASP-1, i.e. that protein segment which is present before the so-called SH3 domain, which begins at amino acid 202.

30

According to recognized principles of interpretation, such an agreement is considered to be a safe identification of the product from the protein spot as a protein corresponding to LASP-1 at least in the range  
5 of the amino acids 1-200. The molecular weight of unphosphorylated LASP-1 (261 amino acids; cf. SEQ ID NO:1) is, however, only 29,717 Dalton. However, it is known from the literature that, in molar mass determinations by gel electrophoresis, LASP-1 appears  
10 to exhibit irregular behaviour so that the deviations found for molar masses are not necessarily in contradiction to the identification of the substance found as LASP-1 with the amino acid sequence according to SEQ ID NO:1.

15

Equally, however, it cannot be completely ruled out that the product detected differs from said LASP-1, for example is the product of an alternative splicing. In order to explore the possibility that it could have  
20 been another related expression product, a subsequent investigation was therefore carried out with the intention of exploring the possibility of assignment of the protein spot found to a closely related longer protein. Subsequent investigations carried out using  
25 information implicitly present in cDNA databases led to the discovery of a longer protein which is related to LASP-1 and is likely to be the product of an alternative splicing and, instead of the SH3 domain, has an alternative C-terminal amino acid residue of 123  
30 amino acids. The protein has the same amino acids 1-200 LASP-1, so that an immunoreactivity comparable with LASP-1 was to be expected. Said related protein was assigned the name LAP-1. Regarding its identification,

further information is to be found in the following section 5.

#### 4. Immunodiagnostic determination of LASP-1 immunoreactivities in sera of human patients

##### 4.1. Immunoassays - Material and methods

##### 4.1.1. Peptide syntheses

10

Three ranges were selected (Pos. 121-137: peptide range 1; Pos. 147-159: peptide range 2; Pos. 170-187: peptide range 3), derived from the known amino acid sequence of human LASP-1. In each case supplemented by an N-terminal cysteine residue, the ranges were chemically synthesized as soluble peptides by standard methods, purified, subjected to quality control by means of mass spectrometry and reversed phase HPLC and lyophilized in aliquots (JERINI AG, Berlin, Germany). The amino acid sequences of the synthetic peptides are:

20

|               |                     |                |
|---------------|---------------------|----------------|
| Peptide PKE18 | CKYHEEF EKSRMGPSGGE | (SEQ ID NO:13) |
| Peptide PPQ14 | CQDGSSYRRPLEQQ      | (SEQ ID NO:14) |
| Peptide PVK19 | CVYQQPQQQPVAQSYGGYK | (SEQ ID NO:17) |

25

As standard peptides, the following peptide which was composed of the positions 121-137, 147-159, 170-187 of LASP-1 according to SEQ ID NO:1 was also synthesized:

|    |              |   |                |
|----|--------------|---|----------------|
| 30 | PeptidePKK54 | KYHEEF EKSRMGPSGGEGGQDGSSYRRPLEQQGGG-VYQQPQQQPVAQSYGGYK | (SEQ ID NO:15) |
|----|--------------|---|----------------|

The following considerations were critical for the



choice of peptides:

The protein LASP-1 comprising 261 amino acids contains two potential protein binding domains, the LIM domain  
5 at the N-terminus (Pos. 5-56) and the SH3 domain at the C-terminus (Pos. 202-261) (cf. Tomasetto et al., loc. cit. 1995). Whether and which protein bindings LASP-1 enters into via these domains is unknown to date. Two possible actin-binding domains are described as further  
10 structural features of LASP-1: these are located directly in succession in the range of Pos. 61-133 (Schreiber et al., loc. cit. 1998), and it has been shown that LASP-1 can actually bind actin (Schreiber et al., loc. cit. 1998). The binding to actin is  
15 influenced by phosphorylation at the positions 99 and 146 (Chew et al. loc. cit., 2002).

For the development of immunoassays for LASP-1 and for examining the question as to whether LASP-1 is also  
20 detectable extracellularly, i.e. as immunoreactivity in normal and pathological human serum/plasma samples, the inventors made use of the known information described above for the structure of LASP-1: three peptides which do not lie in the range of the LIM and SH3 domains were  
25 selected for the immunization, since these domains in LASP-1 might not have been accessible to antibodies owing to bound protein. Furthermore, the three selected proteins do not contain the phosphorylation sites described. For one of the peptides, it was accepted  
30 that it is partly in a possible actin-binding range.

#### 4.1.2. Conjugation and immunization

By means of MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), the peptides PKE18, PQQ14  
5 and PVK19 were each conjugated with the carrier protein  
KLH (keyhole limpet hemocyanine) (cf. working  
instructions "NHS-esters-maleimide crosslinkers" from  
PIERCE, Rockford, IL, USA). Sheep were immunized with  
these conjugates according to the following scheme:  
10 each sheep initially received 100  $\mu$ g of conjugate  
(stated mass based on the peptide content of the  
conjugate) and then 50  $\mu$ g of conjugate every 4 weeks  
(stated mass based on the peptide content of the  
conjugate). Beginning with the fourth month after the  
15 beginning of the immunization, 700 ml of blood per  
sheep were taken every 4 weeks and antiserum was  
obtained therefrom by centrifuging. Conjugations,  
immunizations and recovery of antisera were carried out  
by MicroPharm, Carmarthenshire, UK.

20

#### 4.1.3. Purification of the antibodies

In a 1-step method, the peptide-specific antibodies  
were prepared from the antisera which had been obtained  
25 beginning with the fourth month after immunization.

For this purpose, the peptides PKE18, PQQ14 and PVK19  
were first coupled to SulfoLink Gel (cf. working  
instructions "SulfoLink Kit" from PIERCE, Rockford, IL,  
30 USA). 5 mg of peptide per 5 ml of gel were offered for  
coupling.

The affinity purification of peptide-specific

antibodies from sheep antisera against both peptides was carried out as follows:

The peptide columns were first washed three times alternately with 10 ml each of elution buffer (50 mM citric acid, pH 2.2) and binding buffer (100 mM sodium phosphate, 0.1% Tween, pH 6.8). 50 ml of the antisera were filtered over 0.2  $\mu$ m, and the column material present was added. For this purpose, the gel was rinsed quantitatively with 10 ml of binding buffer from the column. The incubation was effected overnight at room temperature with swirling. The batches were transferred quantitatively into empty columns (NAP 25, Pharmacia, emptied). The run-throughs were discarded. The material was then washed protein-free (protein content of the wash eluate < 0.02 A<sub>280</sub> nm) with 250 ml of binding buffer. Elution buffer was added to the washed columns, and 1 ml fractions were collected. The protein content of each fraction was determined by means of the BCA method (cf. working instructions from PIERCE, Rockford, IL, USA). Fractions having protein concentrations of > 0.8 mg/ml were pooled, neutralized with PBS buffer and thus diluted to protein concentrations of about 1 mg/ml. The yields were: 45 mg of anti-PKE18 antibody, 59 mg of anti-PQQ14 antibody and 14 mg of anti-PVK19 antibody.

#### 4.1.4. Marking

For the chemiluminescent marking of the antibodies, 10  $\mu$ l portions of MA70 acridinium NHS ester (1 mg/ml; from HOECHST Behring) were added to 100 ml of the anti-PVK19 antibody (1 mg/ml) and 125  $\mu$ l of the anti-PQQ14

antibody (0.8 mg/ml) and incubated for 15 minutes at room temperature. Thereafter, 400  $\mu$ l of 1 M glycine were added in each case and incubation was effected for a further 10 minutes. Thereafter, the marking batches  
5 were subjected to a buffer change using NAP-5 gel filtration columns (Pharmacia) in 1 ml each of mobile phase A (50 mM potassium phosphate, 100 mM NaCl, pH 7.4) according to the working instructions and thereby freed from low molecular weight components. For  
10 separating off final residues of labels not bound to antibodies, gel filtration HPLCs were carried out (column: Waters Protein Pak SW300). The samples were applied and were chromatographed at a flow rate of 1 ml/min using mobile phase A. The wavelengths 280 nm  
15 and 368 nm were measured using a flow-through photometer. The absorption ratio 368 nm/280 nm as a measure of the degree of marking of the antibodies was 0.10 for both antibodies. The monomeric fractions containing antibodies (retention time 8-10 min) were  
20 collected, and were collected in 3 ml of 100 mM sodium phosphate, 150 mM NaCl, 5% bovine serum albumin, 0.1% sodium azide, pH 7.4.

#### 4.1.5. Coupling

25

Irradiated 5 ml polystyrene tubes (from Greiner) were coated with anti-PQQ14 antibodies or anti-PKE18 antibodies, as follows: the antibodies were diluted to a concentration of 6.6  $\mu$ g/ml in 50 mM Tris, 100 mM  
30 NaCl, pH 7.8. 300  $\mu$ l of this solution were pipetted into each tube. The tubes were incubated for 20 hours at 22°C. The solution was filtered with suction. Each tube was then filled with 4.2 ml of 10 mM sodium

phosphate, 2% Karion FP, 0.3% bovine serum albumin, pH 6.5. After 20 hours, the solution was filtered with suction. Finally, the tubes were dried in a vacuum dryer.

5

#### 4.2. Carrying out the immunoassays and evaluation thereof

##### 4.2.1. Assay design and procedure

10

An assay buffer having the following composition was prepared:

100 mM sodium phosphate, 150 mM NaCl, 5% bovine serum albumin, 0.1% unspecific sheep IgG, 0.1% sodium azide, pH 7.4

20

A chemically synthesized artificial peptide PKK54, which contains Pos. 121-137, 147-159, 170-187 of LASP-1 (SEQ ID NO:15), served as standard material. This peptide was diluted serially in normal horse serum (from SIGMA). The standards thus prepared were assigned concentrations according to the amount of peptide weighed in.

25

Measurement samples were EDTA plasmas or sera from apparently healthy persons, from patients suffering from sepsis, from patients suffering from Alzheimer's disease and from patients suffering from cardiac infarction.

30

Three types of sandwich assays were carried out. The following antibody combinations were used:

Tube: anti-PKE18 / tracer: anti-PVK19

Tube: anti-PQQ14 / tracer: anti-PKE18

Tube: anti-PQQ14 / tracer: anti-PVK19

5

All assays were carried out as follows:

50  $\mu$ l each of standard or sample and 150  $\mu$ l of assay  
buffer were pipetted into the test tubes. Incubation  
10 was effected for two hours at 22°C with shaking.  
Washing was then effected 4 times with 1 ml of wash  
solution (0.1% Tween 20) per tube each time. 200  $\mu$ l of  
assay buffer, containing 0.5 million RLU of the MA70-  
marked tracer antibody, were then pipetted into each  
15 tube. Incubation was effected for two hours at 22°C  
with shaking. Washing was then effected 4 times with  
1 ml of wash solution (0.1% Tween 20) per tube each  
time, the tube was allowed to drip off and the  
chemiluminescence bound to the tube measured in a  
20 luminometer (from BERTHOLD, LB952T; base reagents  
BRAHMS AG).

Using the MultiCalc software (spline fit), the  
concentration of LASP-1 immunoreactivity was read.

25

#### 4.2.2. Results

Surprisingly, it was found that, with all three  
sandwich assays described above, a substantial and  
30 comparable LASP-1 immunoreactivity was detectable in  
sera of patients suffering from sepsis (cf. Figure 3).  
This immunoreactivity was substantially higher than in  
the case of control samples from healthy persons. The

detected occurrence of LASP-1 immunoreactivity in the blood circulation was surprising against the background of knowledge about LASP-1, since LASP-1 contains no structural features (such as a signal sequence) which would make it possible to predict extracellular localization of the molecule. The prior art, too, provides no indication at all of such possible localization. Rather, intracellular localization in cell membrane extensions is described (Schreiber et al., loc. cit. 1998).

With one of the assays (tube: anti-PQQ14/tracer: anti-PVK19), more extensive measurements were carried out. Dilutions of standard peptide PKK54 in horse serum served for standardization. The substantial increase of LASP-1 immunoreactivity in a larger number of cases of sera from patients suffering from sepsis (Figure 4) was confirmed. An immunoreactivity likewise increased compared with healthy controls was also detected for patients suffering from Alzheimer's disease (Figure 5) and, to a somewhat lesser extent, for patients suffering from cardiac infarction (Figure 6).

#### **4.2.3. Information on protein binding of LASP-1 in serum**

The signal intensities for the individual serum samples - approximately proportional to the analyte concentration in the sandwich immunoassay - correlated between three different assays (Figure 3). This correlation shows that all three epitopes are accessible in a similar manner for the binding of the antibodies used in all measured samples. For an analyte

which is not bound to other proteins, this would be expected and not surprising. For LASP-1, however, several possibilities have been described for binding to proteins (loc. cit.). One of the epitopes (Pos. 121-137) overlaps with an actin binding site. Evidently, this binding site is therefore not occupied with LASP-1 immunoreactivity in the analyte measurable in this serum. The other two epitopes (Pos. 147-159 and Pos. 170-187) are outside protein binding domains (actin-binding sites, LIM, SH3). A direct influence on antibody binding at these isotopes by bound proteins is therefore not to be expected. However, the possibility of the occurrence of indirect steric hindrance of the antibody binding in the case of protein binding cannot be ruled out. However, this is evidently not the case. Whether the LASP-1 immunoreactivity in the serum is actually freely present has not yet been proved by the present analyses and may have to be determined by further investigations.

20

#### **5. Identity of the LASP-1 immunoreactivity in serum**

A comparison of the amino acid sequences of the three peptides PKE18 (Pos. 121-137 in LASP-1; SEQ ID NO:13), PQQ14 (Pos. 147-159 in LASP-1; SEQ ID NO:14) and PVK19 (Pos. 170-187 in LASP-1; SEQ ID NO:17) used for producing the anti-LASP-1 antibodies with the sequences entered in the GenBank database for all known human proteins or proteins derived from cDNAs showed that, in addition to LASP-1 (e.g. Accession No. BC012460), a second protein which contains these sequences exists (Accession No. BC007560). This is referred to below as LAP-1. It can be assigned the sequence according to

30



SEQ ID NO:16. The immunoreactivity measured by the immunoassays described above could therefore originate both from LASP-1 (SEQ ID NO:1) itself and from LAP-1 (SEQ ID NO:16).

5

The Applicant has not become aware of any prior art relating to LAP-1 over and above the entries on the primary structure in the database. In order to obtain more detailed information on the structure and genesis  
10 of LASP-1 in comparison with LAP-1, the inventors themselves therefore carried out more extensive analyses, the results of which are summarized below:

LASP-1 and LAP-1 evidently result from different  
15 splicing of the primary transcript of one and the same gene: the sequences of the cDNAs of LASP-1 and LAP-1 (Accession No. BC007560 and BC012460) were compared with the sequence of the human genome. An associated gene was localized at chromosome 17, close to 17q21,  
20 region 39.023K-39.076K. By comparison of the sequences, it was possible to derive an exon-intron structure of the gene. The cDNAs for LASP-1 and LAP-1 result from the splicing of eight exons. Exons 2-5 do not differ for the two cDNAs. However, exons 6,7 and 8 are not  
25 identical in the two cDNAs but overlap one another. Exon 1 differs in the two cDNAs with regard to the length at the 5' terminus. What remains unclear is whether the different lengths of exon 1 of the two cDNAs result from different transcription initiations  
30 or from different breaks in the reverse transcription of the mRNAs. The translated region extends for both cDNAs from the 3' region into the 5'-region of exon 7: the amino acid sequences of the two translation

products are identical up to the amino acid proline (Pos. 200), but completely different thereafter (cf. SEQ ID NO:1 in comparison with SEQ ID NO:16). LASP-1 comprises 261 amino acids altogether, and LAP-1  
5 comprises 323 amino acids. In LASP-1, the SH3 domain begins at position 202. It is precisely this SH3 region which is not present in LAP-1 but is replaced by another region of unknown function. The lack of the SH3 domain in LAP-1 led to its naming (in "LASP-1", S  
10 represents SH3 domain).

Both for LASP-1 and for LAP-1, ESTs (expressed sequence tags) are present in the relevant GenBank "Human EST entries" database (e.g. BU553748 for LASP-1 and  
15 BG281978 for LAP-1). This clearly shows that the entries described above, BC007560 and BC012460, are not singular observations or even artefacts but rather indicate that both splicing variants are actually expressed. However, the conditions under which the gene  
20 is expressed in LASP-1 or LAP-1 are unknown.

On the basis of the substantial sequence identity with LASP-1, a determination and/or codetermination of LAP-1 is also to be regarded as a determination according to  
25 the present invention.

If, for an assay method, the reagents are chosen so that the assay is capable of distinguishing between LASP-1 and LAP-1, for example by choosing the binding  
30 partners (solid phase-bound or marked) in a sandwich assay in such a way that at least one of them specifically recognizes an epitope in the C-terminal part above position 200 of LAP-1 or LASP-1, in

particular above position 200 of LAP-1, it is possible to check whether the LASP-1 immunoreactivity detected in a biological fluid is completely or partly attributable to LAP-1, and LAP-1 can also be determined  
5 alone alongside LASP-1, or alongside the total amount of LASP-1 and LAP-1, and the clinical relevance of the presence of LAP-1 for sepsis and/or Alzheimer's disease can thus be explored more exactly.

#### 10 **Therapeutic potential of the novel discoveries**

The function of the greatly increased LASP-1 immunoreactivity observed under pathological conditions in the blood circulation is unclear. However, simply  
15 the circumstance that an increase is associated with a pathological state suggests that the LASP-1 immunoreactivity is involved in the molecular mechanism of the disease or in the endogenous attempt to fight the disease, and possibly even plays a key role  
20 thereby. It is therefore promising to carry out further investigations based on the discoveries in the present Application and with a therapeutic objective. Depending on whether the LASP-1 immunoreactivity tends to be harmful or useful, therapeutic benefits will arise  
25 either through an exogenous reduction or increase in the LASP-1 immunoreactivity.

The increased LASP-1 or possibly LAP-1 concentrations found for the first time permit their use as novel  
30 biomarkers for sepsis and inflammations, in particular infectious inflammations, and Alzheimer's disease and also in association with cardiac diseases, in particular in cardiac infarction and other severe

cardiovascular diseases and provisionally also in cancer. The occurrence of LASP-1 during the disease in the circulation of patients suffering from sepsis, Alzheimer's disease and cardiac infarction, which has  
5 been proved for the first time, furthermore opens up novel interesting research aspects for the therapy of the relevant diseases.